Supporting Information:

In Vivo Chemiluminescent Imaging Agents for Nitroreductase and Tissue Oxygenation

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1. Synthetic procedures

General Methods. All reactions were performed in dried glassware under an atmosphere of dry N_2. Silica gel P60 (SiliCycle) was used for column chromatography and SiliCycle 60 F254 silica gel (precoated sheets, 0.25 mm thick) was used for analytical thin layer chromatography. Plates were visualized by fluorescence quenching under UV light or by staining with iodine. Other reagents were purchased from Sigma-Aldrich (St. Louis, MO), Alfa Aesar (Ward Hill, MA), EMD Millipore (Billerica, MA), Oakwood Chemical (West Columbia, SC), and Cayman Chemical (Ann Arbor, MI) and used without further purification. ^{1}H NMR and ^{13}C NMR spectra for characterization of new compounds and monitoring reactions were collected in $CDCl_3$, D_2O, or DMSO-d6 (Cambridge Isotope Laboratories, Cambridge, MA) on a JEOL 500 MHz spectrometer in the Department of Chemistry at Southern Methodist University. All chemical shifts are reported in the standard notation of parts per million using the peak of residual proton signals of the deuterated solvent as an internal reference. Coupling constant units are in Hertz (Hz). Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; dt, doublet of triplets. High-resolution mass spectroscopy was performed on a Shimadzu IT-TOF (ESI source) and low-resolution mass spectroscopy was performed on a Shimadzu LCMS-8050 Triple Quadrupole LCMS (ESI source) or a Shimadzu Matrix Assisted Laser Desorption/Ionization MS (MALDI) at the Shimadzu Center for Advanced Analytical Chemistry at the University of Texas, Arlington. Compound **1** was synthesized according to a previously published procedure.[1]

4-nitrobenzyl (2,5-dioxopyrrolidin-1-yl) carbonate (2). *N*,*N*'-Disuccinimidyl carbonate (1253 mg, 4.891 mmol, 1.5 equiv) was added to a solution of 4-nitrobenzyl alcohol (500 mg, 3.26 mmol, 1.0 equiv) in 10.0 mL CH_2Cl_2, followed directly by the addition of NEt_3 (1.37 mL, 9.79 mmol, 3.0 equiv). The reaction was stirred for 8.5 h at rt. The reaction was quenched with 20 mL 1 M $NaHCO_3$, extracted with 2 x 30 mL CH_2Cl_2, washed with 10 mL brine, dried over Na_2SO_4, filtered, and concentrated to yield **2** (955.3 mg) as an orange oil and used without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.26 (d, 2H, J = 8.6 Hz), 7.57 (d, 2H, J = 8.6 Hz), 5.40 (s, 2H), 2.83 (s, 4H).

5-(((1*r*,3*r*,5*R*,7*S*) - adamantan - 2 -ylidene) (methoxy) methyl)-2-chlorophenyl (4-nitrobenzyl) carbonate (3). Phenol **1** (235 mg, 0.77 mmol, 1.0 equiv) was dissolved in 5 mL 4:1 THF:CH_2Cl_2 in a dry flask under N_2 atmosphere. Mixed carbonate **2** (284 mg, 0.92 mmol, 1.2 equiv) was added as a solution in 1.5 mL CH_2Cl_2. DMAP (146 mg, 1.2 mmol, 1.5 equiv) and NEt_3 (322 µL, 2.31 mmol, 3.0 equiv) were then added in succession. After 10 h of stirring at rt, the mixture was poured into a separatory funnel containing 20 mL CH_2Cl_2 and 15 mL DI-H_2O and extracted with 3 x 20 mL CH_2Cl_2. The organic layer was washed with 10 mL brine, dried over Na_2SO_4, filtered, and concentrated. Purification by silica column chromatography (1:15 EtOAc/hexanes) afforded **3** as a clear oil (164.8 mg, 44%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, 2H, J = 8.6 Hz), 7.60 (d, 2H, J = 8.6 Hz), 7.39 (d, 1H, J = 8.0 Hz), 7.19 (m, 2H), 5.39 (s, 2H), 3.28 (s, 3H), 3.22 (s, 1H), 2.63 (s, 1H), 1.20–2.00 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.57, 148.09, 146.76, 141.86, 141.66, 136.03, 134.00, 130.04, 128.60,

(1) Cao, J.; Lopez, R.; Thacker, J. M.; Moon, J. Y.; Jiang, C.; Morris, S. N. S.; Bauer, J. H.; Tao, P.; Mason, R. P.; Lippert, A. R. *Chem. Sci*. **2015**, *6*, 1979–1985.

128.45, 125.35, 124.00, 123.74, 69.06, 58.14, 39.21, 39.08, 37.10, 32.30, 30.44, 28.24; HRMS calcd for $C_{26}H_{26}NO_6Cl$ $(M+Na)^+$ 484.1521, found 484.1519.

2-chloro-5-((1*r*,3*r*,5*r*,7*r*)-4'-methoxyspiro[adamantane-2,3'-[1,2]dioxetan]-4'-yl)phenyl (4-nitrobenzyl) carbonate (HyCL-1). Enol ether **3** (80 mg, 0.17 mmol, 1.0 equiv) and rose bengal (8 mg, 0.0079 mmol, 0.046 equiv) were added into a dry flask and dissolved in 7 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 °C and the residue was purified by silica column chromatography (1:15 EtOAc/hexanes) to deliver **HyCL-1** as a white solid (68 mg, 75%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.27 (d, 2H, *J* = 8.6 Hz), 7.61 (d, 2H, *J* = 8.6 Hz), 7.00–7.80 (br, 3H), 5.40 (s, 2H), 3.21 (s, 3H), 3.01 (s, 1H), 2.06 (s, 1H), 1.00–1.90 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 152.41, 148.17, 146.73, 141.65, 135.57, 130.45, 128.62, 128.12, 124.07, 111.05, 95.48, 69.18, 50.17, 36.33, 34.89, 33.23, 33.05, 32.26, 31.80, 31.60, 26.01, 25.88.

(1*r*,3*r*,5*R*,7*S*) -2 - ((4-chloro-3-((4-nitrobenzyl) oxy) phenyl) (methoxy) methylene) adamantane (5). Phenol **1** (230 mg, 0.68 mmol, 1.0 equiv) and triphenylphosphine (214 mg, 0.82 mmol, 1.2 equiv) were dissolved in anhydrous THF. Diethyl azodicarboxylate (128 μL, 0.82 mmol, 1.2 equiv) was added dropwise over 5 min and then 3-nitrobenzyl alcohol (104 mg, 0.68 mmol, 1.0 equiv) was added immediately. After 1 h of stirring at rt, the mixture was concentrated. Purification by silica column chromatography (1:12 EtOAc/hexanes) afforded **5** as a yellow oil (250 mg, 84 %). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.24 (d, 2H, *J* = 8.6 Hz), 7.65 (d, 2H, *J* = 8.6 Hz), 7.35 (d, 1H, *J* = 8.0 Hz), 6.91 (m, 2H), 5.25 (s, 2H), 3.26 (s, 3H), 3.21 (s, 1H), 2.54 (s, 1H), 1.20–2.00 (m, 12H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.33, 147.65, 144.01, 142.42, 135.54, 132.97, 129.97, 127.50, 123.90, 123.48, 122.21, 114.54, 69.42, 57.90, 39.23, 39.06, 37.13, 32.38, 30.35, 28.27; HRMS calcd for $C_{25}H_{26}NO_4Cl$ $(M-H)^-$ 438.1478, found 438.1466.

(1*r*,3*r*,5*r*,7*r*)-4'-(4-chloro-3-((4-nitrobenzyl)oxy)phenyl)-4'-methoxyspiro [adamantane-2,3'-[1,2]dioxetane] (HyCL-2). Enol ether **5** (75 mg, 0.17 mmol, 1.0 equiv) and rose bengal (8.5 mg, 0.0084 mmol, 0.049 equiv) were added into a dry flask and dissolved in 5 mL THF. Oxygen was bubbled through the reaction mixture, while irradiating with a 120 W light bulb (Home Depot, Dallas, TX) at 0 °C. After 3 h of reaction, TLC showed no starting material left and the mixture was then concentrated under vacuum at 0 °C and the residue was purified by the silica column chromatography (1:15 EtOAc/hexanes) to deliver **HyCL-2** as a white solid (56.4 mg, 71%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.25 (d, 2H, *J* = 8.6 Hz), 7.67 (d, 2H, *J* = 8.6 Hz), 6.80–7.30 (br, 3H), 5.34 (s, 2H), 3.24 (s, 3H), 2.98 (s, 1H), 2.06 (s, 1H), 1.00–2.00 (m, 13H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 153.33, 147.74, 143.68, 134.98, 127.56, 124.00, 111.57, 95.57, 87.03, 69.48, 50.04, 36.31, 34.83, 33.28, 33.17, 32.28, 31.72, 31.54, 25.97, 25.90; HRMS calcd for $C_{25}H_{26}NO_6Cl$ $(M+Na)^+$ 494.1341, found 494.1337.

2. Chemiluminescent response to nitroreductase and NADH

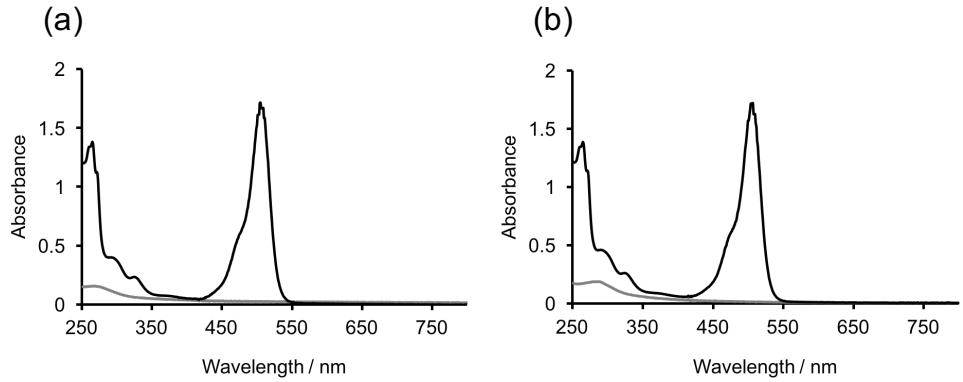

Figure S1. Absorption spectra of (a) 10 μM **HyCL-1** and (b) 10 μM **HyCL-2** with 10% Emerald II Enhancer (black) or without Emerald II Enhancer (grey) in 10 mM PBS buffer.

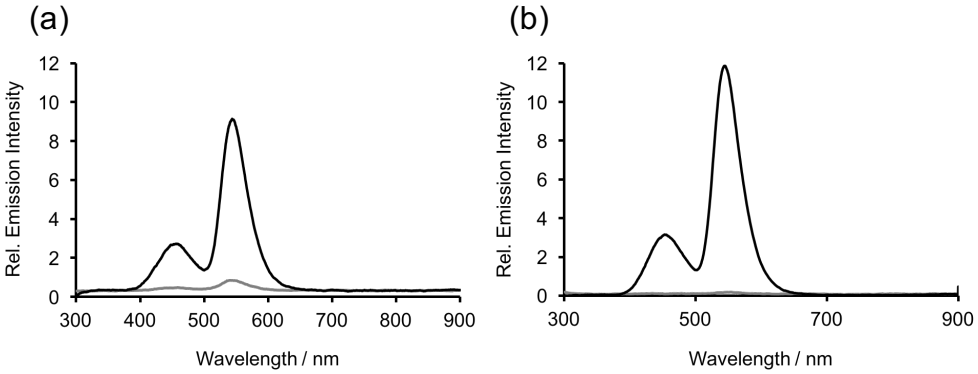

Figure S2. Chemiluminescent emission spectra of (a) 10 μM **HyCL-1** and (b) 10 μM **HyCL-2** with 0 (grey) or 14 (black) μg/mL nitroreductase (NTR) in the presence of 0.4 mM NADH in 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer acquired 30 min after adding NTR.

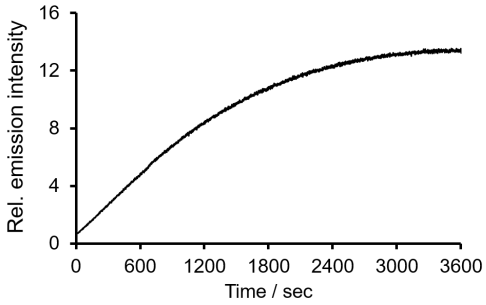

Figure S3. Long time scan of the chemiluminescence emission at 545 nm of 10 μM **HyCL-2** to 14 μg/mL NTR and 0.4 mM NADH in 10 mM PBS buffer (pH 7.4) and 10% Emerald II Enhancer.

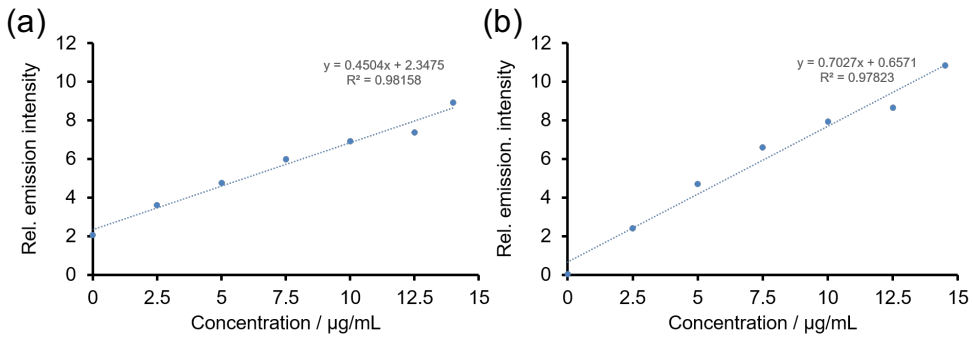

Figure S4. Chemiluminescent emission at 545 nm from (a) 10 µM **HyCL-1** and (b) 10 µM **HyCL-2** and 0, 2.5, 5, 7.5, 10, 12.5, 14 µg/mL nitroreductase in the presence of 0.4 mM NADH in 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer.

3. GC-MS analysis of the reaction of HyCL-2 with nitroreductase

2966 µL of a 10 mM PBS buffer (pH 7.4), 6 µL of 10 mM **HyCL-2** in DMSO, 24 µL of 50 mM NADH in 0.01 mM NaOH, 4 µL of 10 mg/mL NTR were added into a vial, mixed well. After 3 h of incubation at rt, the mixture was poured into a separatory funnel containing 10 mL CH_2Cl_2 and 15 mL DI-H_2O and extracted with 3 x 10 mL CH_2Cl_2. The organic layer was collected, dried over Na_2SO_4, filtered, and concentrated. Then the solid was re-dissolved in 2 mL CH_2Cl_2, transferred to a GC-MS vial and GC-MS was conducted immediately using a 6850 Series GC-MS (Agilent Technologies, Santa Clara, CA). As a control experiment, the same procedure was conducted except that **HyCL-2** was replaced with 6 µL DMSO. Mass spectra were averaged across the peak found in the extracted ion chromatogram for m/z = 186 (Figure S5), m/z = 150 (Figure S6), and m/z = 121 (Figure S7). Matches to the NIST database were found using the NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library Version 2.0 d, build Dec 2, 2005 installed with the Enhanced Chemstation software.

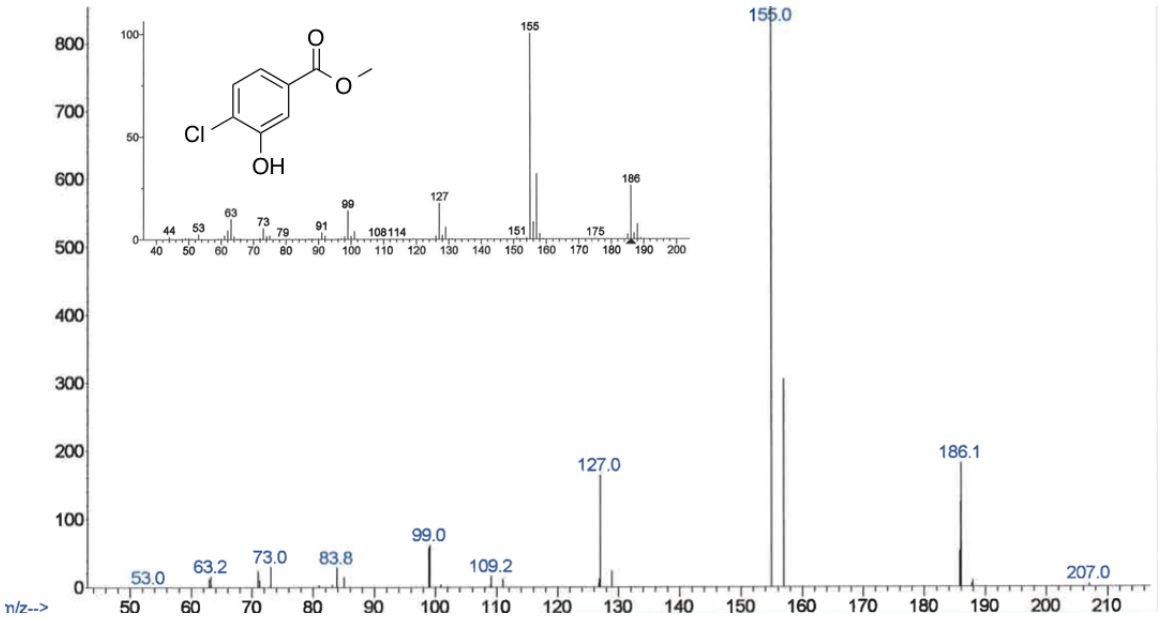

Figure S5. Mass spectrum for the peak found in the extracted chromatogram at m/z = 186. Inset is the mass spectra found by NIST Mass Spectral Search Program.

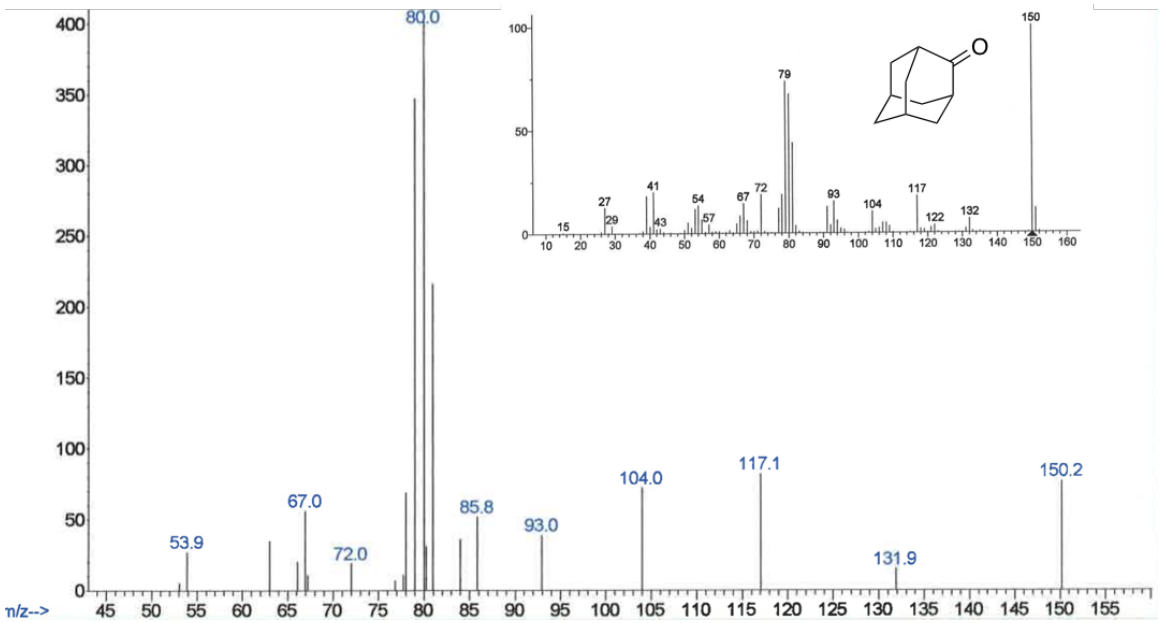

Figure S6. Mass spectrum for the peak found in the extracted chromatogram at m/z = 150. Inset is the mass spectra found by NIST Mass Spectral Search Program.

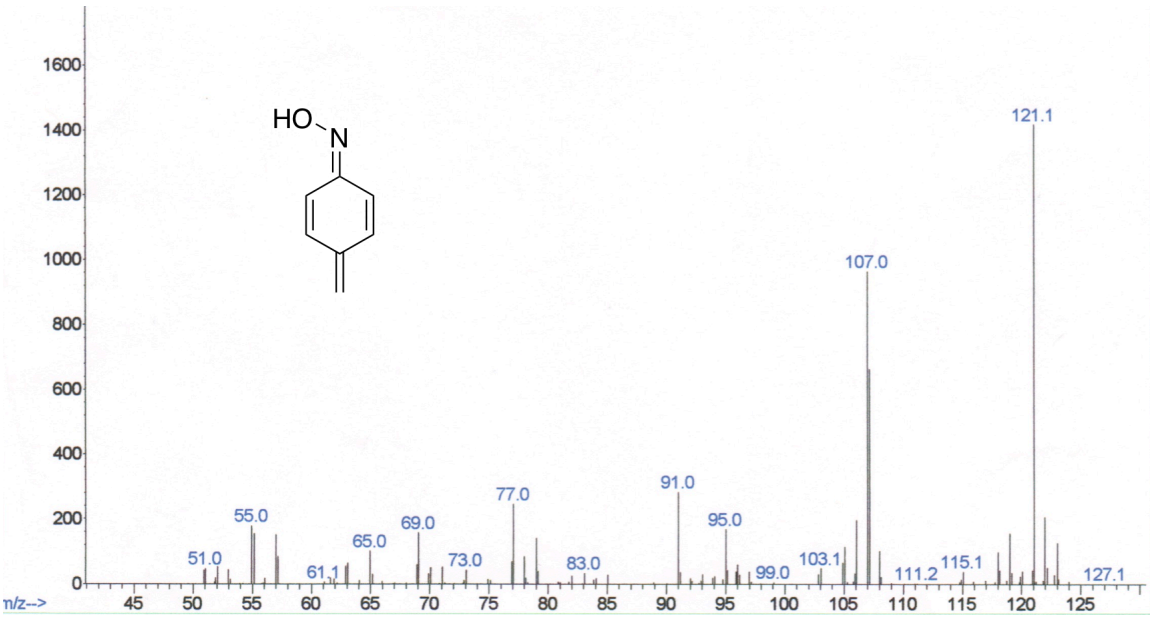

Figure S7. Mass spectrum for the peak found in the extracted chromatogram at m/z = 121.

4. Monitoring reaction of HyCL-1 and nitroreductase by ^{1}H NMR

500 µL of 10 mM **HyCL-1** in DMSO-d_6 and 100 µL of 100 mM cysteine in D_2O were mixed well in an Eppendorf tube and then transferred to an NMR tube. The reaction progress was monitored by ^{1}H NMR at 500 MHz.

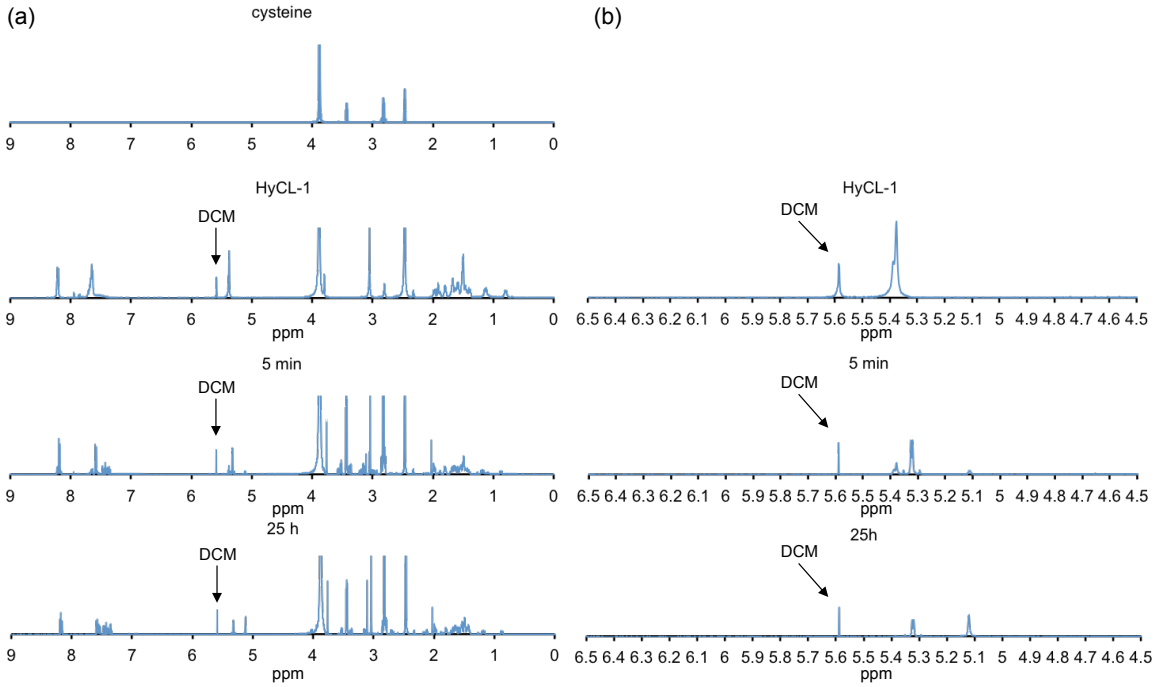

Figure S8. ^{1}H NMR spectra of 8.33 mM **HyCL-1** and 16.67 mM cysteine in 5:1 DMSO-d^6:D_2O. (a) Full ^{1}H NMR spectra of cysteine, **HyCL-1**, and 5 min and 25 hours after adding reagents to the NMR tube. (b) Expansion of the ^{1}H NMR spectra from 4.5 to 6.5 ppm of **HyCL-1** and 5 min and 25 hours after adding reagents to the NMR tube.

5. Detailed procedures for selectivity tests

NTR (14 μg/mL) and NADH (0.4 mM): 889 μL of a 10 mM PBS buffer (pH 7.4), 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO, 8 μL of 50 mM NADH in 0.01 mM NaOH, 1.4 μL of 10 mg/mL NTR, and 100 μL Emerald II Enhancer were added to an Eppendorf tube and was shaken gently to assure mixing. The mixed solution was then transferred to a quartz cuvette.

Glutathione (5 mM): 50 μL of a 100 mM stock solution of glutathione in DI-H_2O was added to a solution of 848 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

L-Cysteine (1 mM): 10 μL of a 100 mM stock solution of L-cysteine in DI-H_2O was added to a solution of 888 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

Homocysteine (1 mM): 10 μL of a 100 mM stock solution of homocysteine in DI-H_2O was added to a solution of 888 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

Dithiothreitol (200 μM): 2 μL of a 100 mM stock solution of dithiothreitol in DI-H_2O was added to a solution of 896 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

NADH (0.4 mM): 890 μL of a 10 mM PBS buffer (pH 7.4), 2 μL of a 5 mM **HyCL-1** or **HyCL-2** in DMSO, 8 μL of a 50 mM NADH, and 100 μL Emerald II Enhancer were added to an Eppendorf tube and was shaken gently to assure mixing. The mixed solution was then transferred to a quartz cuvette.

NTR (14 μg/mL): 897 μL of a 10 mM PBS buffer (pH 7.4), 2 μL of a 5 mM **HyCL-1** or **HyCL-2** in DMSO, 1.4 μL of NTR, and 100 μL Emerald II Enhancer were added to an Eppendorf tube and was shaken gently to assure mixing. The mixed solution was then transferred to a quartz cuvette.

H_2S (200 μM): 10 μL of a 20 mM stock solution of Na_2S in DI-H_2O was added to a solution of 888 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

Sodium Citrate (200 μM): 2 μL of a 100 mM stock solution of sodium citrate in DI-H_2O was added to a solution of 896 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

$Na_2S_2O_5$ (200 μM): 2 μL of a 100 mM stock solution of $Na_2S_2O_5$ in DI-H_2O was added to a solution of 896 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

L-ascorbic acid (200 μM): 2 μL of a 100 mM stock solution of L-ascorbic acid in DI-H_2O was added to a solution of 896 μL 10 mM PBS buffer (pH 7.4) and 2 μL of 5 mM **HyCL-1** or **HyCL-2** in DMSO and then 100 μL Emerald II Enhancer was added into this mixture in an Eppendorf tube and then transferred to a quartz cuvette.

Blank: 2 µL of 5 mM **HyCL-1** or **HyCL-2** in DMSO was added to a solution of 898 µL 10 mM PBS buffer (pH 7.4) and then 100 µL Emerald II Enhancer was added.

6. Deoxygenation experiments

Gas bubbling experiments. 10 mM PBS buffer (pH 7.4) and Emerald II Enhancer were mixed in a ratio of 9 to 1 in a 250 mL round bottom flask and 100% O_2, air, or N_2 was bubbled through it for 60 min. Then 989 µL of this solution was transferred into a capped cuvette by syringe, and 2 µL of a 5 mM **HyCL-2** in DMSO, 8 µL of a 50 mM NADH in 0.01 mM NaOH solution, 1.25 µL of nitroreductase (1 mg nitroreductase dissolved in 100 µL DI-H_2O) were added into the cuvette using a syringe. Time scans were initiated 1 min after adding nitroreductase (Figure S9).

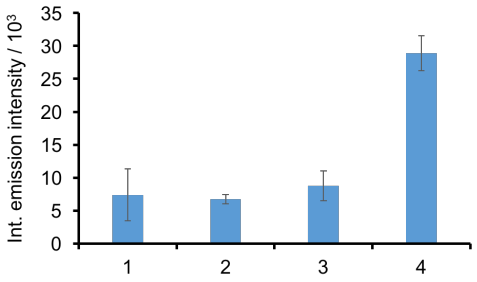

Figure S9. Integrated chemiluminescent emission at 545 nm from (1) ambient conditions, (2) 100% O_2 bubbled, (3) air bubbled, or (4) N_2 bubbled 10 mM PBS buffer (pH 7.4) containing 10% Emerald II Enhancer with 12.5 µg/mL of nitroreductase in the presence of 0.4 mM NADPH and 10 µM **HyCL-2** integrated over 20 min. Error bars are ± S.D.

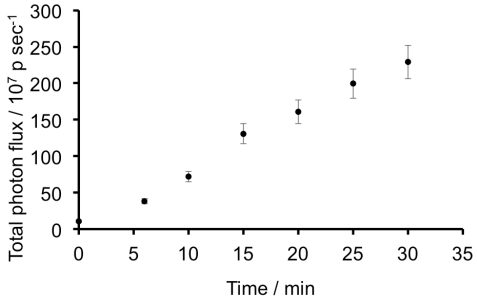

Figure S10. Time-dependence of imaging of nitroreductase using **HyCL-2**. Images were taken 3.45 min after adding 10 µM **HyCL-2** to 12.5 µg/mL NTR in 10 mM PBS buffer (pH 7.4) containing 0.4 mM NADH and 10% Emerald II Enhancer at different time point (n = 3 wells). Error bars are ±S.D.

7. Scanned ^{1}H and ^{13}C NMR spectra

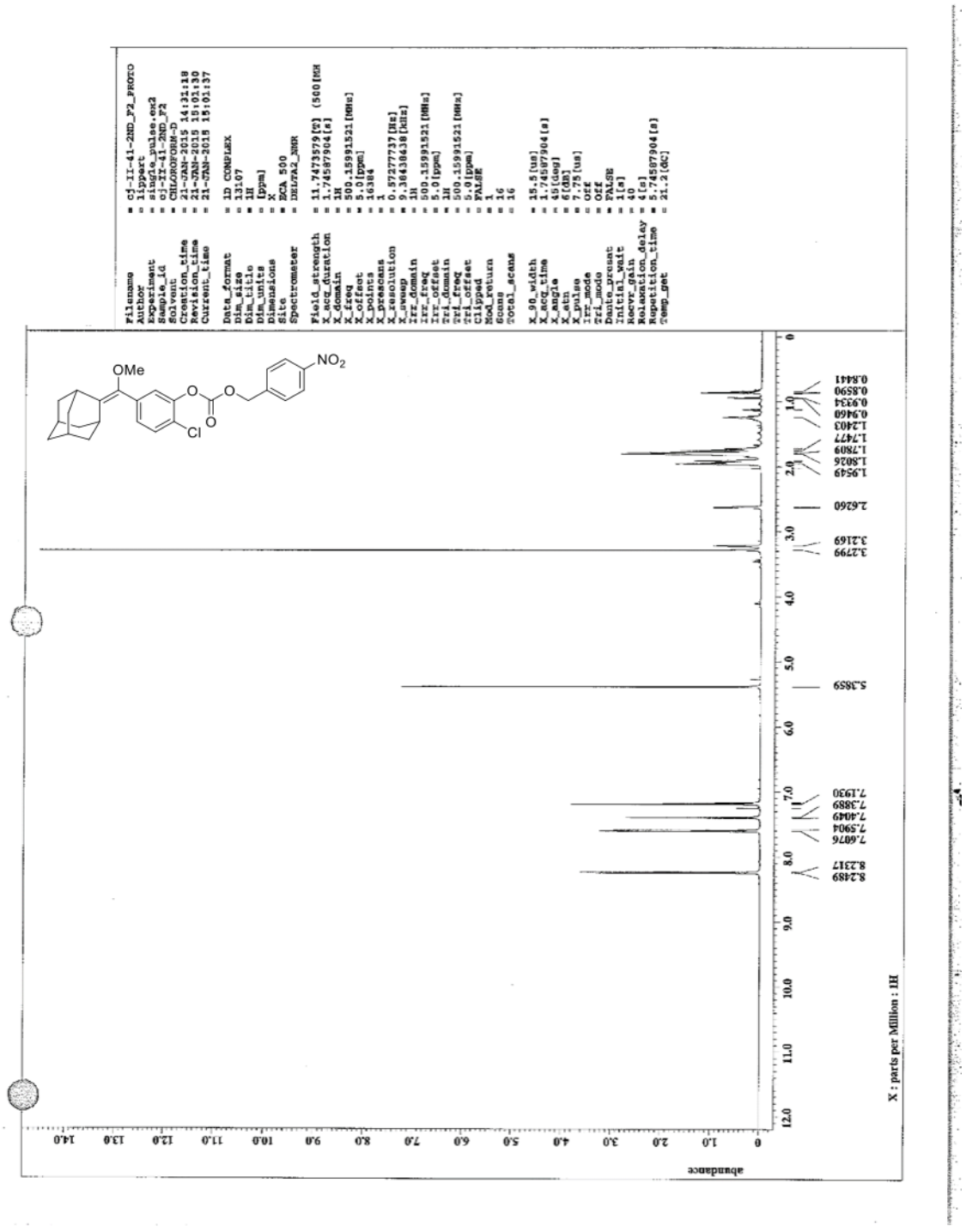

Figure S11. ^{1}H NMR (500 MHz, $CDCl_3$) of **3**.

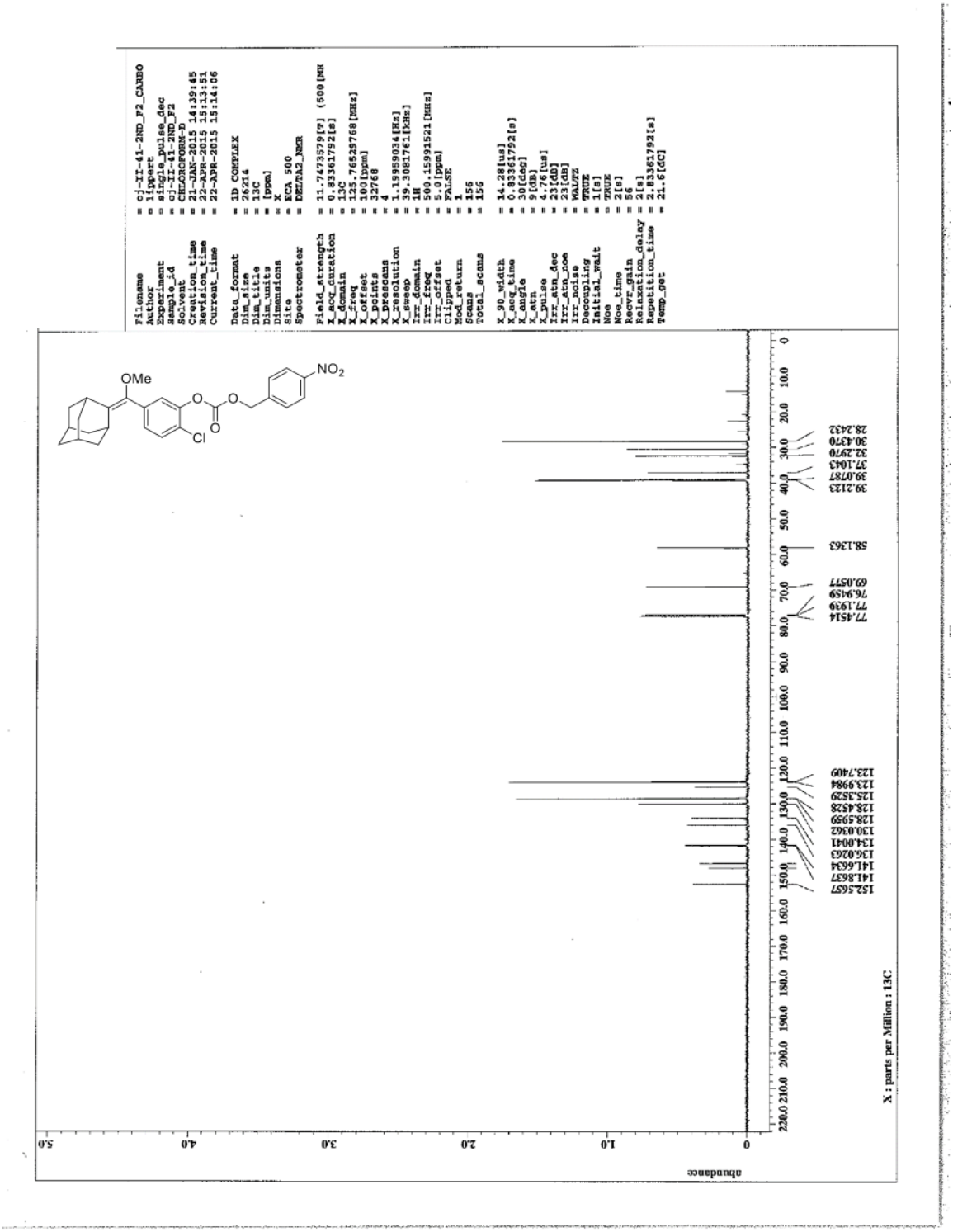

Figure S12. ^{13}C NMR (500 MHz, $CDCl_3$) of **3**.

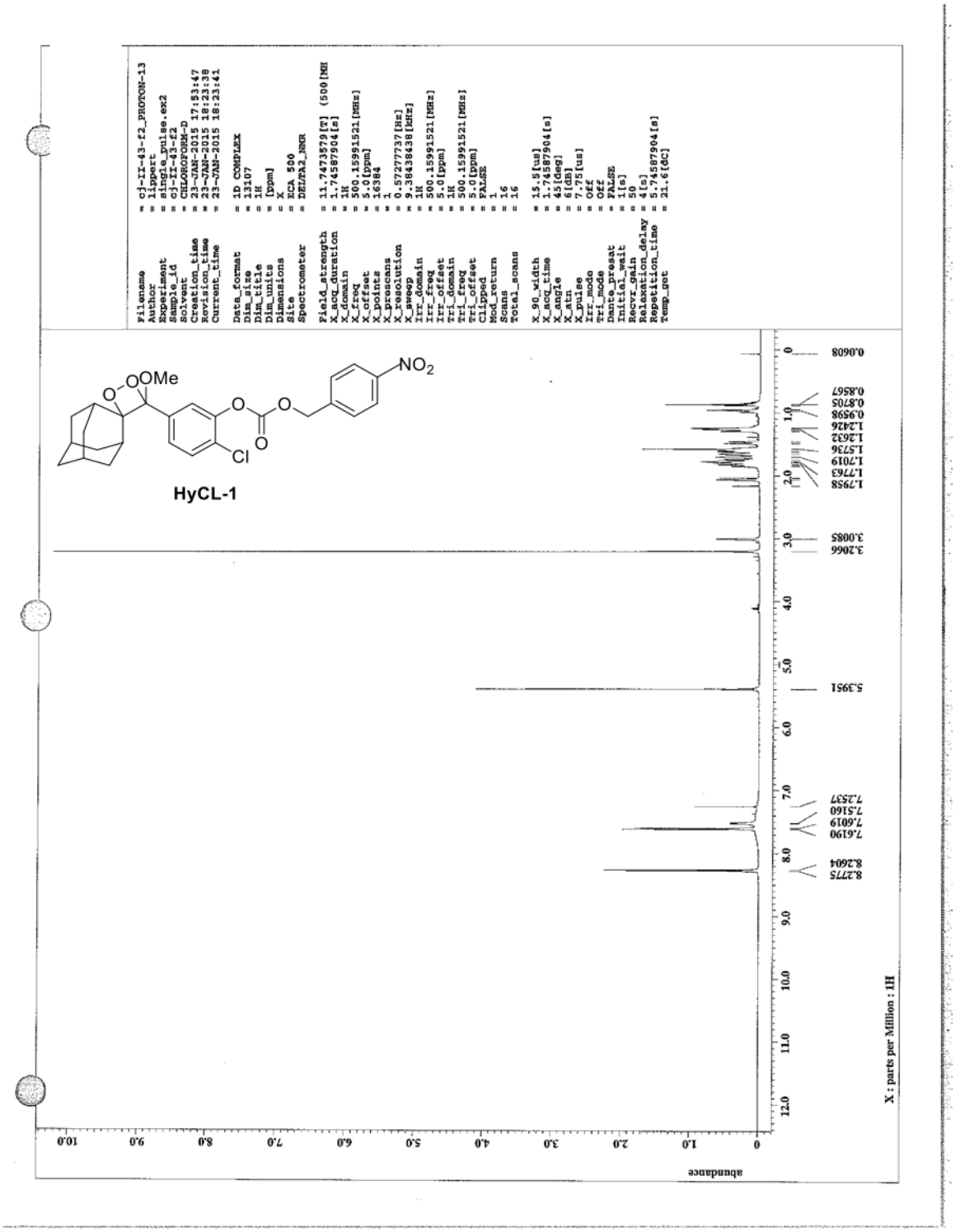

Figure S13. ^{1}H NMR (500 MHz, $CDCl_3$) of **HyCL-1**.

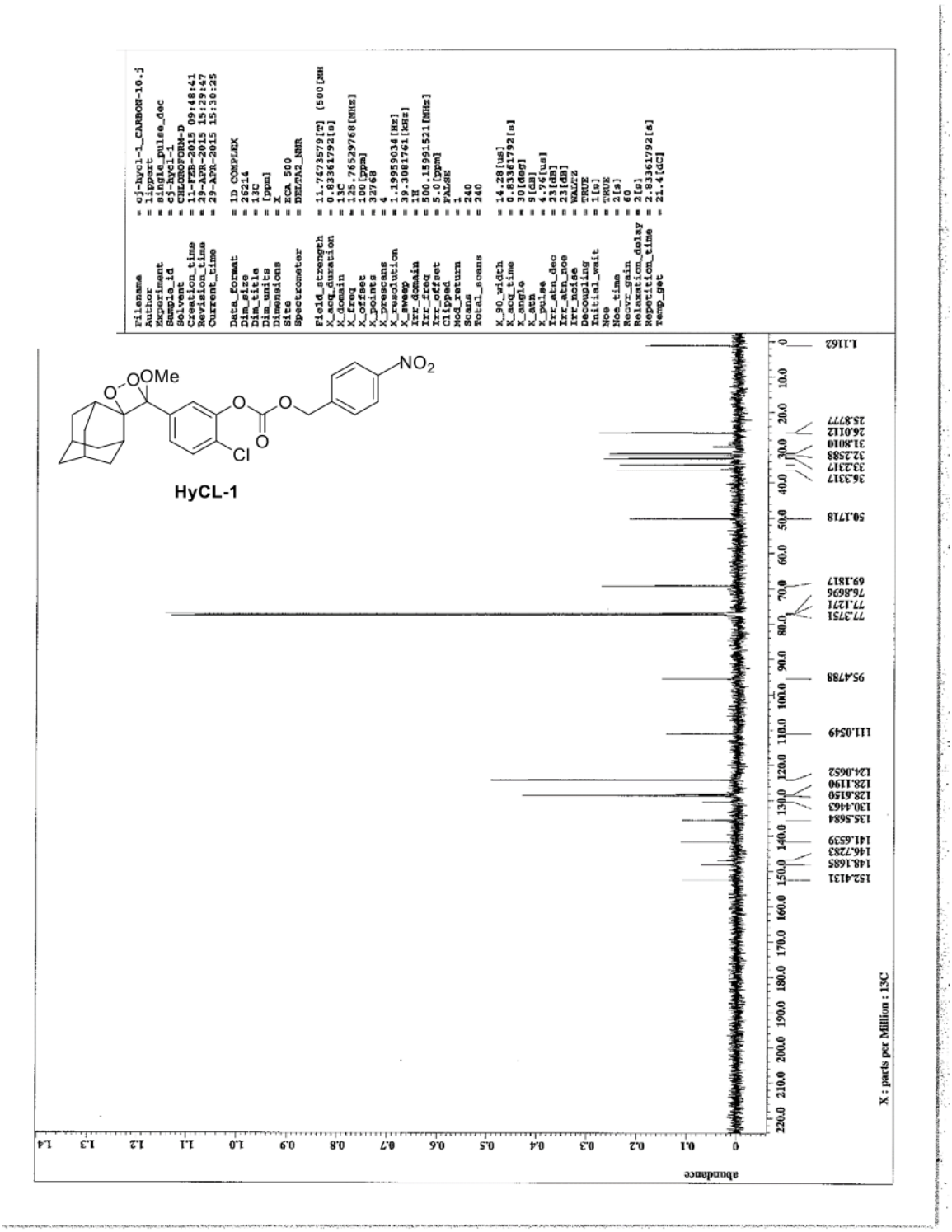

Figure S14. ^{13}C NMR (500 MHz, $CDCl_3$) of **HyCL-1**.

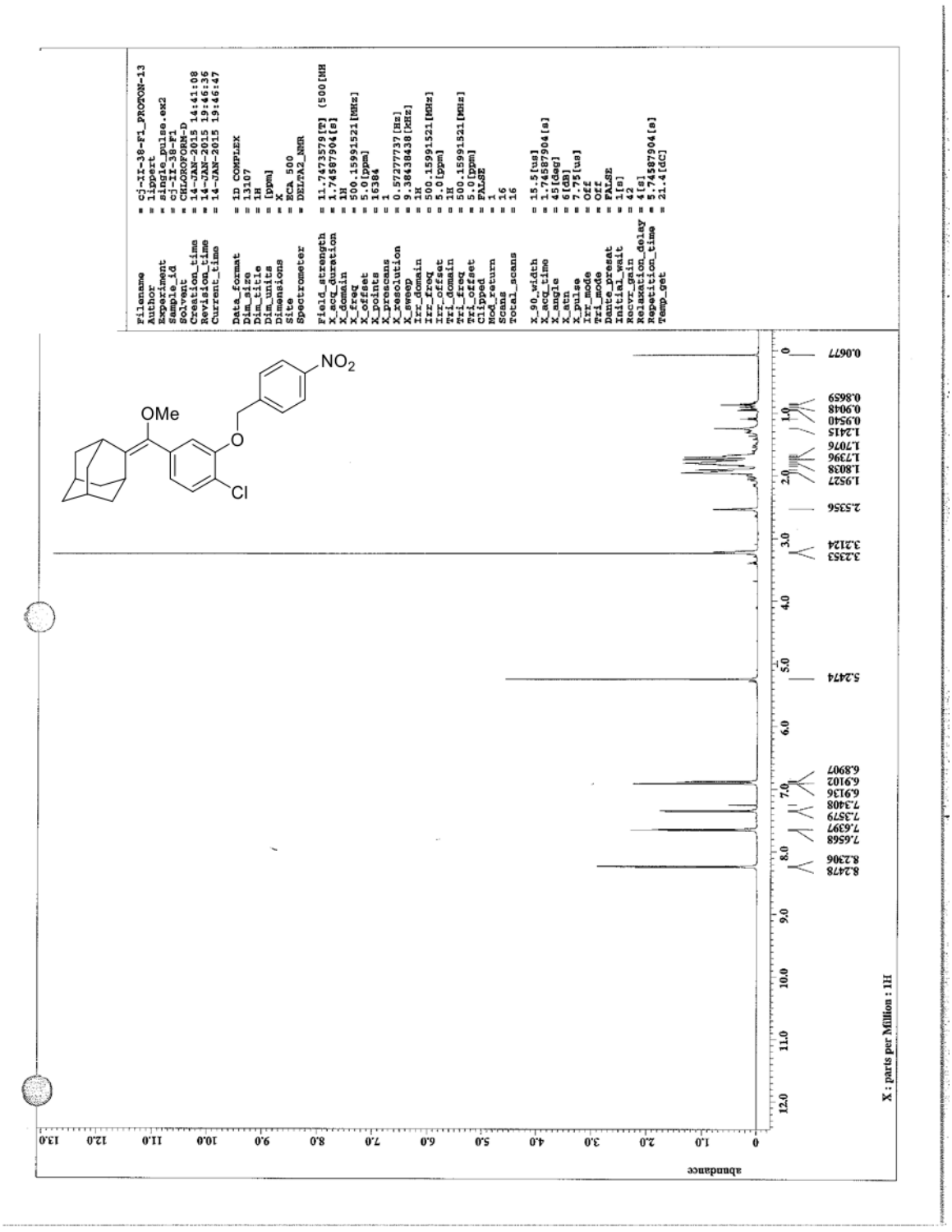

Figure S15. ^{1}H NMR (500 MHz, $CDCl_3$) of **5**.

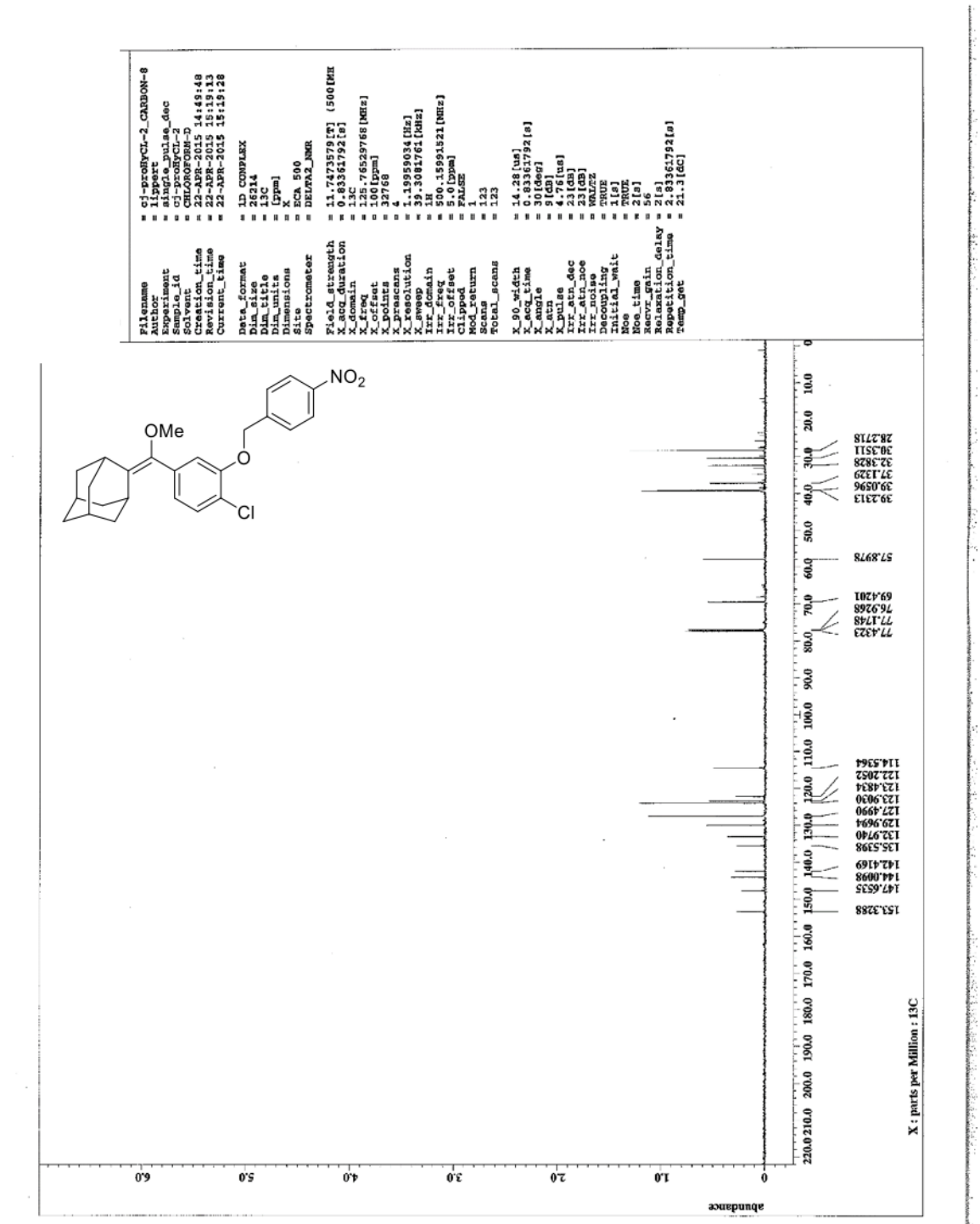

Figure S16. ^{13}C NMR (500 MHz, $CDCl_3$) of **5**.

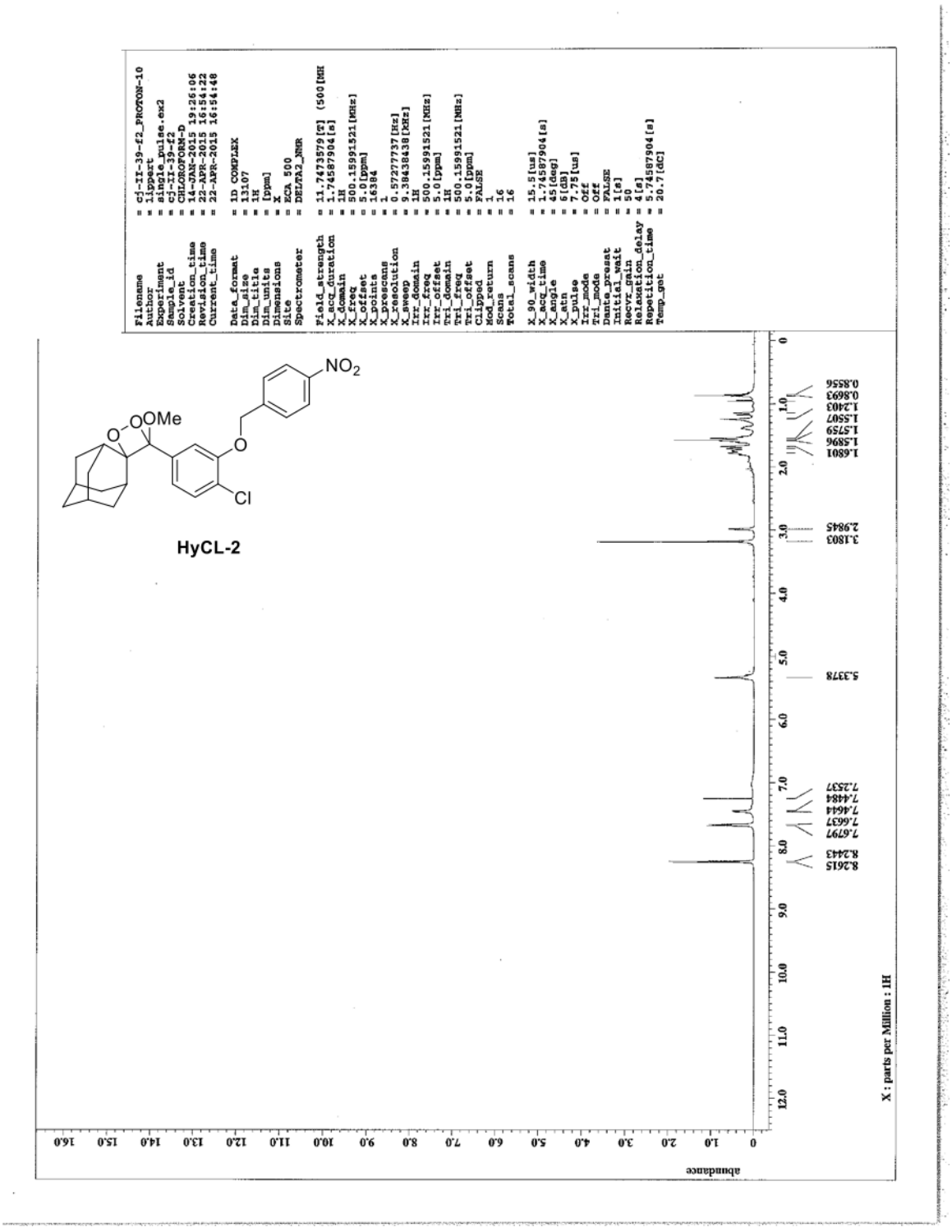

Figure S17. ^{1}H NMR (500 MHz, $CDCl_3$) of **HyCL-2**.

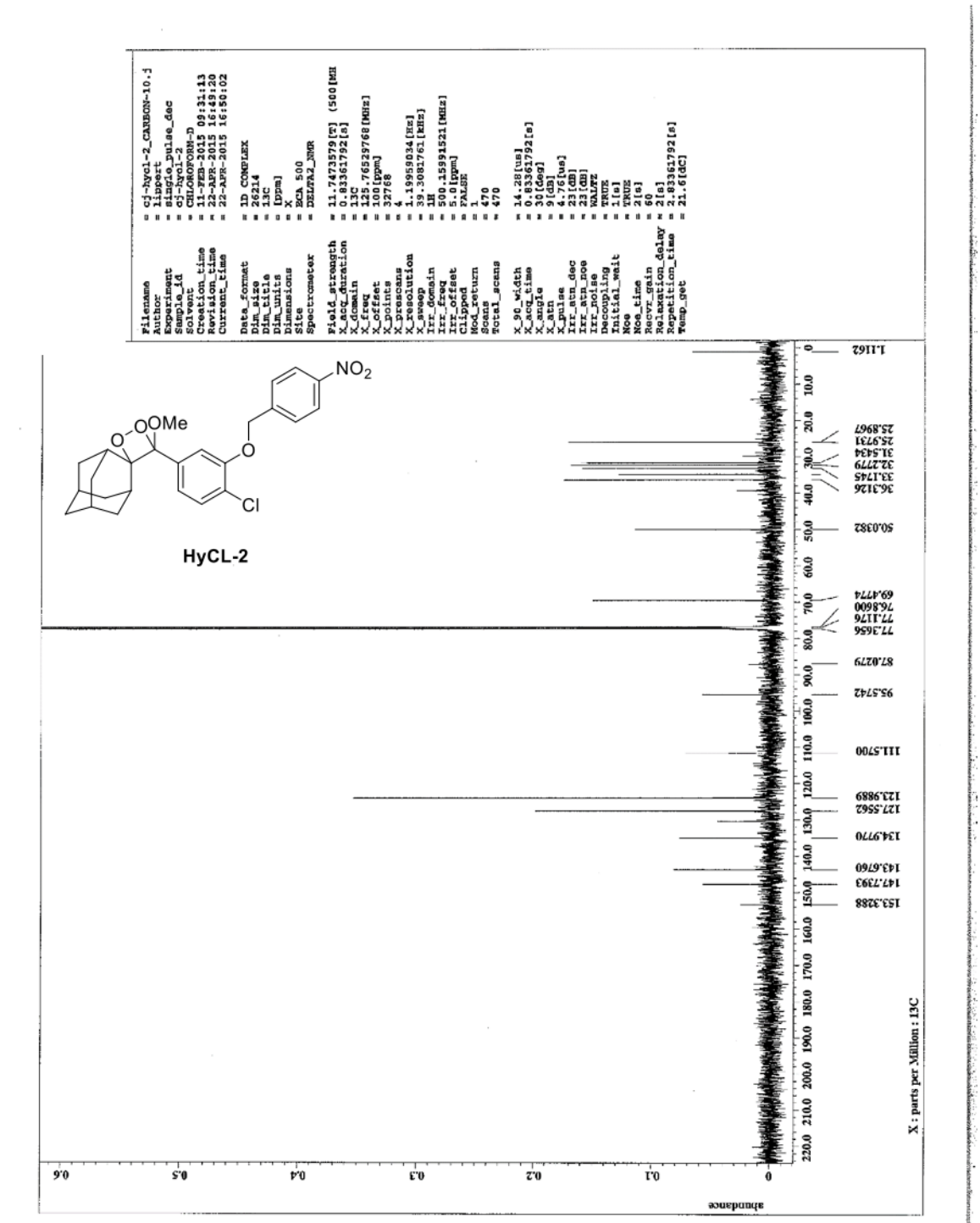

Figure S18. ^{1}H NMR (500 MHz, $CDCl_3$) of **HyCL-2**.